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RESEARCH ARTICLE

HEPATOPROTECTIVE EFFECT OF TRIPHALA AND ITS COMBINATIONS- IN HEPG$_2$ CELL LINES.

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Abstract

Introduction: From ancient period onwards Triphala and its combinations were used in liver disorders in Ayurveda, but its mechanism of action are not still evaluated. Triphala is the combination in equi-proportion 1:1:1, of dried fruits of Amalaki (Phyllanthus emblica Linn), Bibhitaki (Terminalia bellerica Roxb) and Haritaki (Terminalia chebula Retz). The present study evaluated the hepatoprotective effect of methanolic extract of Triphala (T) and its different combinations such as Triphala with honey [TM], Triphala with Pippali (dried fruit of Piper longum Linn) [TP] and Triphala with Yashtimadhu (rhizhomes of Glycyrrhiza glabra Linn) [TY] in HepG$_2$ cell lines using MTT assay against acetaminophen induced hepato toxicity. Flow cytometry was used to count the viable cells.

Methodology: HepG$_2$ (Human hepatic carcinoma) cell line was purchased from NCCS Pune and maintained in Dulbecco's modified eagle's media. Then T, TM, TP and TY were added in to acetaminophen induced hepatotoxicity cells at five concentrations; 6.25µg/ml, 12.5 µg/ml, 25 µg/ml, 50 µg/ml and 100 µg/ml using MTT assay [(3-(4,5 dimethylthiazole-2yl)-2,5-diphenyl tetrazolium bromide assay]; untreated cells and acetaminophen treated cells kept as controls.

Results: The acetaminophen treated cells showed 34.15% viability in MTT assay. The T, TM, TP and TY treated groups showed increase in the percentage cell viability at 6.25µg/ml, 12.5 µg/ml, 25 µg/ml, 50 µg/ml and 100 µg/ml concentrations and the result were significant ($p<0.05$), when compared to acetaminophen treated group. In flow cytometry total apoptosis was 28.88% for T,39.04% for TM ,42.04% for TP and 44.12% for TY.

Conclusion:The results validate that Ayurveda combination T has more hepato protective effect in HepG$_2$ cell line; in the increasing order TY< TP < TM < T.

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Introduction:-

Liver is the organ for metabolism, excretion and maintenance of homeostasis of the body[1]. On continuous exposure to environmental toxins, chemicals like CCl_4 , alcohol, drug habits, infections and autoimmune disorders will lead to various pathological manifestation of liver[2].These factors enhance lipid peroxidation and other oxidative changes in the liver. Hepatic pathology is one among the fatal diseases and it accounts for approximately 2 million deaths per year worldwide[3]. Herbal medicines have a lot to do with the alleviation of liver disorders [4]. According to the estimate more than 700 mono or poly herbal formulations in the form of decoction, tincture, tablets and capsule are known as hepatoprotective[5]. Triphala is the one such combination (equal proportion 1:1:1) of dried fruits (Phala) of Amalaki (Phyllanthus emblica Linn), Bibhitaki (Terminalia bellirica Roxb) and Haritaki (Terminalia chebula Retz). In Ayurveda, it is commonly used to balance the three doshas (humors) in the body, viz. Vatta, Pitta and Kapha[6].Traditionally, the Triphala formulation has been used as a laxative in chronic constipation, as a detoxifying agent of the colon, and as a rejuvenator of the body[7]. According to recent researches, the Triphala formulation also shows antidiabetic[8] and hepatoprotective activities and plays an important role in blood pressure control and balances cholesterol[9]. Triphala also used in combination with honey or Pippali (fruit of Piper longum Linn), or Yastimadhu (Glycirhizha glabra Linn) in various liver disorders. The present work intended to study the hepatoprotective activity of methanolic extracts of Triphala (T), Triphala with honey (TM) , Triphala with Pippali(TP) and Triphala with Yashtimadhu (TY) against acetaminophen induced toxicity using $HepG_2$ cell lines.

Materials And Methods:-

Plant material :-

The Triphala (combination of equiproportion of Terminalia chebula Retz, Terminalia bellirica Roxb and Phyllanthus emblica Linn) , Pippali (Piper longum Linn) and Yashtimadhu (Glycyrrhiza glabra Linn) were purchased from local market was authenticated by pharmacognosy unit, Govt. Ayurveda college , Trivandrum. The plant material was dried at room temperature, pulverized by a mechanical grinder, sieved through 85 mesh, stored in an air tight and light resistant container for further use. Genuineness was assured by preliminary pharmacognostical screening, macroscopical evaluation of fruit rind of Terminalia chebula Retz(Haritaki), Terminalia bellirica Roxb (Vibhitaki) and Phyllanthus emblica Linn(Amalaki), Pippali (Piper longum Linn) and Yashti madhu (Glycyrrhiza glabra Linn) and microscopic evaluation of its powder.

Preparation of the extract:-

10g of fine powder of Triphala (T), 5g each of T and honey (M), 5g each of T and Pippali (P) and 5g each of T and Yashtimadhu (Y) were taken and to each of these 4 samples (T, TM,TP and TY) 25 ml of methanol was added and separately reflexed for 2 hours in Liebig condenser. After that they were separately filtered through filter paper and were evapourated to dryness using water bath to get solid residue.

Reagents: -

Acetaminophen (20µmol), Trypsin, Fetal Bovine Serum (FBS), Palmitic acid, oil red, MTT(3-(4,5-dimethyl thiazolyl)-2,5-diphenyl-tetrazolium bromide, Dimethyl Sulfoxide (DMSO), Dulbecco's Modified Eagles medium (DMEM),glutamine, streptomycin, penicillin and Amphotericin B.

Cell lines and maintenance: -

$HepG_2$ (Human hepatic carcinoma) cell line was purchased from NCCS Pune and maintained in Dulbecco's modified eagle's media (DMEM) (Sigma Aldrich USA) containing L-glutamine with high glucose.

Cell culture and sub culturing of $HepG_2$ cells: -

The $HepG_2$ cell line was cultured in 25 cm^2 tissue culture flask with DMEM supplemented with 10% FBS, L-glutamine, sodium bicarbonate and antibiotic solution containing Penicillin (100U/ml), Streptomycin (100µg/ml) and Amphotericin B (2.5µg/ml). Cultured cell lines were kept at 37˚C in a humidified 5% CO_2 incubator (NBS Eppendorf, Germany)[10]. The viability of cells was evaluated by direct observation of cells by inverted phase contrast microscope and followed by MTT assay method. Two days old confluent monolayer of cells were trypsinized and the cells were suspended in 10% growth medium,100µl cell suspension (5×10^4 cells/well) was seeded in 96 well tissue culture plate and incubated at 37˚C in a humidified 5% CO_2 incubator[11]. One mg of the study drug was weighed and completely dissolved in 1ml DMEM using a cyclomixer. The extract solution was filtered through 0.22 µm Millipore syringe filter to ensure the sterility. Acetaminophen (20µmol) was added to induce toxicity[12,13].

After attaining sufficient growth, Acetaminophen (20µmol) was added to induce toxicity and incubated for one hour, prepared extracts in 5% DMEM were five times serially diluted by two-fold dilution (100µg, 50µg, 25µg,12.5 µg, 6.25 µg) in 500µl of 5% DMEM and each concentration of 100µl were added in triplicates to the respective wells and incubated at 37˚C in a humidified 5% CO_2 incubator. Entire plate was observed at an interval of each 24 hours; up to 72 hours in an inverted phase contrast tissue culture microscope (Olympus CKX41 with Optika Pro5 CCD camera) and microscopic observation were recorded as images. Any detectable changes in the morphology of the cells, such as rounding or shrinking of cells, granulation and vacuolization in the cytoplasm of the cells were considered as indicators of cytotoxicity[14].

MTT assay: -

MTT is a colorimetric assay that measures the reduction of yellow 3-(4, 5dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, coloured (dark purple) formazan product. The cells are then solubilized with an organic solvent (isopropanol) and the released, solubilized formazan reagent. Since reduction of MTT can only occur in metabolically active cells the level of activity is a measure of the viability of the cells [15].

15 mg of MTT (Sigma, M-5655) was reconstituted in 3 ml Phosphate Buffered Saline (PBS) until completely dissolved and sterilized by filter sterilization. After 24 hours of incubation period, the sample contents in the wells were removed and 30 µl of reconstituted MTT solution was added to all test and cell control wells, the plate was gently shaken well, then incubated at 37˚C in a humidified 5% CO_2 incubator for 4 hours[16]. After the incubation period, the supernatant was removed and 100 µl of MTT solubilization solution (DMSO) was added and the wells were mixed gently by pipetting up and down in order to solubilize the formazan crystals. The absorbance values were measured by using microplate reader at a wave length of 540 nm (Laura B. Talarico et al, 2004)

The percentage of growth inhibition was calculated using the formula:

$$\text{Percentage of viability} = \frac{\text{Mean OD samples} \times 100}{\text{Mean OD of control group}}$$

Statistical analysis for MTT assay: -

Group comparison made for Statistical analysis was determined by using one-way ANOVA. By comparing with acetaminophen induced hepatotoxicity, the hepatoprotective effect of T at concentrations of 12.5, 25 and 50µg/ml was significant with P value of $P<0.05$. In the case of TM, its hepatoprotective effect was statistically significant at concentrations of 50 µg/ml and 100 µg/ml and having a P value of $P< 0.05$. In case of TP, its hepatoprotective effect was statistically significant with concentration of 25 µg/ml and having a P value of $P< 0.05$. In the case of TY, its hepatoprotective effect was statistically significant at concentrations of 50 µg/ml and 100 µg/ml with a P value of $P< 0.05$.

Flow cytometry:-

Flow cytometry is a sophisticated instrument measuring multiple physical characteristics of a single cell such as size and granularity simultaneously as the cell flows in suspension through a measuring device. Its working depends on the light scattering features of the cells under investigation, which may be derived from dyes or monoclonal antibodies targeting either extracellular molecules located on the surface or intracellular molecules inside the cell [17]. One of the application of this technique is the analysis of apoptosis. Apoptosis is a carefully regulated process of cell death that occurs as a normal part of development. Apoptosis is distinguished from necrosis or accidental cell death by characteristic morphological and bio chemical changes, including compaction and fragmentation of chromatin, shrinkage of cytoplasm, and loss of membrane asymmetry.

Procedure

$HepG_2$ cells were cultured as per standard procedure described earlier. After attaining sufficient growth, Acetaminophen (20 µmol) was added to induce toxicity and incubated for one hour and treated with selected concentration of drugs [T (25µmol), TM (50 µmol), TP (25 µmol) and TY (100 µmol)] and incubated for 24 hours. The cells were trypsinized after incubation and 100 µl of cells in suspension was transferred to separate tubes. To the tubes added 100 µl of Muse™ and Annexin V and dead cell reagent to each tube. The tubes were mixed thoroughly by pipetting up and down or vortexing at a medium speed for 3-5 seconds followed by incubation for 20 minutes at room temperature in the dark. The cells were analyzed in a flow cytometer and analyzed using Muse flow cytometry software. Cells were gated against untreated control cells and analyzed for apoptosis using Muse FCS[18].

Result:-

Acetaminophen mediated hepatotoxicity was taken here as the experimental model for liver injury. Acetaminophen is commonly used hepatotoxic in the experimental study of free radical induced liver diseases. In MTT assay, T showed a cell viability of 60.66% at 25 µg/ml, TM showed a cell viability of 67.26% at 50 µg/ml, TP showed a cell viability of 62.22% at 25 µg/ml and TY showed a viability of 61.5% at 100 µg/ml.

But in flow cytometry the results were as follows.

In acetaminophen treated $HepG_2$ cells the percentage of apoptosis was 50.08%, while it was reduced to 28.88%,39.0%, 42.04%, and 44.12% on treatment with T, TM, TP and TY respectively. On analyzing with MTT assay results, TM was more hepatoprotective than T. But in flow cytometry, the T was more hepatoprotective than TM. This may be due to the fact that the MTT assay was confounded by the reduction of MTT reagent by honey's reducing sugars and phenolic compounds [19]

Table 1:-Effect of T, TM, TP and TY on Invitro Hepatoprotective activity by MTT assay

Concentration(µg/ml)	OD I	OD II	OD III	Average OD	Percentage of viability
Control	0.6297	0.5893	0.5293	0.5828	100
Acetaminophen	0.1898	0.2087	0.1985	0.1990	34.15
T					
6.25	0.2249	0.231	0.1492	0.2017	34.61
12.5	0.2453	0.281	0.3503	0.2922	50.14
25	0.2544	0.3779	0.4282	0.3535	60.66
50	0.3358	0.3338	0.3229	0.3308	56.77
100	0.2948	0.2298	0.239	0.2545	43.67
TM					
6.25	0.2627	0.3155	0.2181	0.2654	45.54
12.5	0.2884	0.3207	0.2103	0.2731	46.87
25	0.3779	0.3258	0.2506	0.3181	54.58
50	0.561	0.3713	0.2436	0.3920	67.26
100	0.5575	0.3227	0.236	0.3721	63.84
TP					
6.25	0.2613	0.2868	0.297	0.2817	48.34
12.5	0.3237	0.3047	0.2988	0.3091	53.03
25	0.4086	0.3617	0.3176	0.3626	62.22
50	0.3219	0.263	0.3066	0.2972	50.99
100	0.2337	0.2418	0.219	0.2315	39.72
TY					
6.25	0.2162	0.2085	0.2252	0.2166	37.17
12.5	0.2361	0.3106	0.2263	0.2577	44.21
25	0.2548	0.3471	0.2922	0.2980	51.14
50	0.291	0.3768	0.3281	0.3320	56.96
100	0.3568	0.3706	0.3479	0.3584	61.50

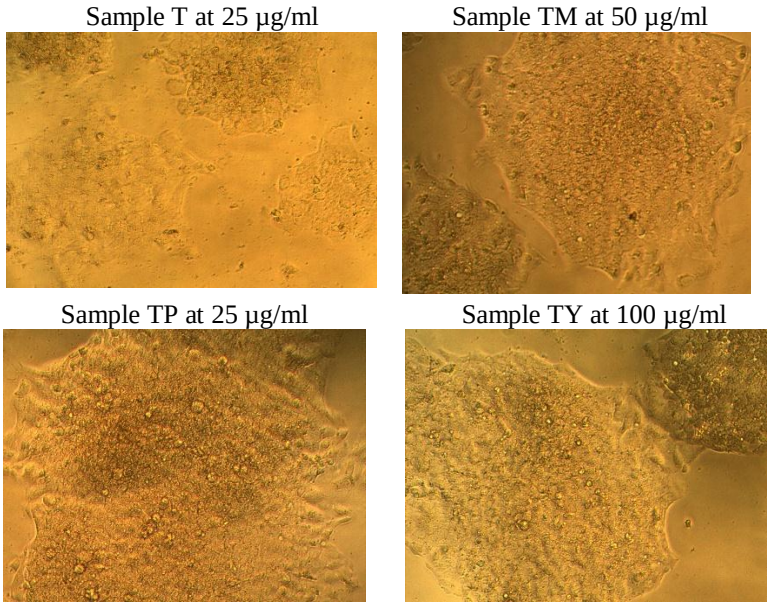

Fig1:- Cell viability of T,TM,TP and TY on $HepG_2$ cells

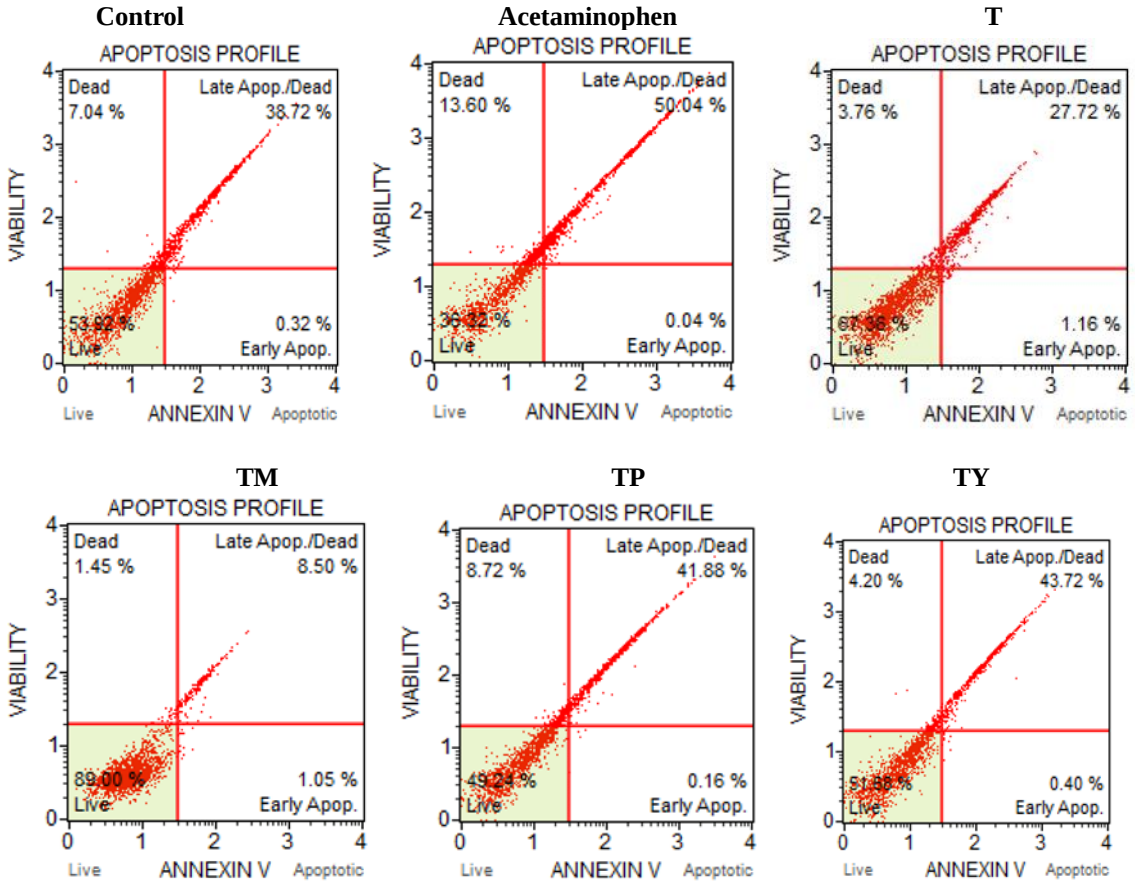

Fig 2:-Apoptosis profile for invitro hepatoprotective activity

Discussion:-

Triphala has been used since ancient times to treat wide range of disease. It is extensively used to treat hepatic disorders and even possesses anti diabetic, anti-hypertensive, anti dyslipidaemic, anti oxidant activities. It possesses different phytochemicals with different biological activity. Different phytochemicals like saponins, tannins, flavonoid and polyphenols present in the Triphala may help in protecting various chronic diseases[20]. It was reported that tannins and flavonoids present in Phyllanthus emblica Linn, high content of phenolic compounds and flavonoids present both in Terminalia chebula Retz and Terminalia bellirica Roxb might be responsible for the hepatoprotective activity of Triphala[21]. Pippali (Piper longum Linn) also possess anti diabetic, hypocholestraemic, hepatoprotective, and antioxidant properties[22]. Glycyrrhizin, a triterpene glycoside from root of Yashtimadhu(Glycyrrhiza glabra Linn), has positive effects on inhibition of hepatic apoptosis and necrosis by suppression of TNF-α and caspase-3, an important cytokine, which is a key mediator of hepatic apoptosis and necrosis in LPS/D-GaAlN-induced liver failure and downregulation of matrixmetalloproteinase-9 in lipopolysaccharide/ D-galactosamine-induced liver injury[23]. Since the plants possess various useful active principles, it was proposed to study the hepatoprotective potential of its combinations by invitro method. In MTT assay for the evaluation of cell viability of methanolic extracts of T, TM, TP and TY, it was observed that the TM showed maximum hepatoprotective activity against $HepG_2$ cells. But in flow cytometry the drug T showed maximum hepatoprotective effect. This may be due to the fact that the MTT assay was confounded by the reduction of MTT reagent by honey's reducing sugars and phenolic compounds. By analyzing MTT assay results with flow cytometry, it was proven that the drug T has maximum hepatoprotective activity than its other combinations with M, P and Y.

Conclusion:-

The present study reveals the hepatoprotective activity of T, TM, TP, and TY by in vitro analysis on $HepG_2$ cells against acetaminophen induced toxicity which was proven by MTT assay and flow cytometry. The results validate that Ayurveda combination T has more hepato protective property; in the increasing order TY< TP < TM < T. Further works are to be carried out to isolate and identify the active principles involved in the hepatoprotective activity of the afore said extracts.

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Conflict of Interests: -
Declared none.

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